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Role of caspases (ICE/CED 3 proteases) in DNA damage and cell death in response to a mitochondrial inhibitor, antimycin A

Rapid Communication

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Role of caspases (ICE/CED 3 proteases) in DNA damage and cell death in response to a mitochondrial inhibitor, antimycin A. Caspases (ICE/Ced3 proteases) are a closely related family of cysteine proteases that play a key role in apoptotic cell death. We examined the role of caspases in DNA damage and cell death in response to the mitochondrial inhibitor, antimycin A. LLC-PK_1 cells contain caspase activity that was markedly inhibited by cleavage site-based peptide inhibitors of caspases but not by inhibitors of serine, cysteine, aspartate or metalloproteinases. The caspase activity increased within five minutes of exposure to antimycin A, preceding any evidence of DNA damage and cell death. The specific caspase inhibitors, Ac-Tyr-Val-Ala-Asp-aldehyde (inhibitor I) and Ac-Asp-Glu-Val-Asp-aldehyde (inhibitor II) prevented, in a dose dependent manner, antimycin A-induced DNA strand breaks as determined by DNA unwinding assay (residual double stranded DNA in control, 94 ± 2%; antimycin A alone, 48 ± 3%; antimycin A + inhibitor I at 50 μM, 93 ± 2%; antimycin A + inhibitor II at 50 μM, 89 ± 5%; $N = 3$ to 4, $P < 0.001$). These inhibitors also prevented antimycin A-induced DNA fragmentation as determined by agarose gel electrophoresis and by *in situ* labeling of cell nuclei by the terminal deoxynucleotidyl transferase (TdT) nick end labeling (TUNEL) method. The caspase inhibitors markedly prevented antimycin A-induced cell death in a dose-dependent manner as measured by trypan blue exclusion (control 6 ± 1%, antimycin A alone 40 ± 1%, antimycin A + inhibitor I at 50 μM 16 ± 1%, antimycin A + inhibitor II at 50 μM 16 ± 1%; $N = 4$ to 7, $P < 0.001$). These data indicate that the caspase family of enzymes play an important role in DNA damage and cell death in response to the mitochondrial inhibitor, antimycin A.

Interleukin 1 β-converting enzyme (ICE) and its family of proteases (ICE-like) have been identified as a group of closely related cysteine proteases involved in apoptosis [1–5]. Recently the trivial name "caspase" has been suggested as the root for serial names for all family members of ICE/Ced-3 proteases [6]. The selection of the name "caspase" includes two distinct catalytic properties of these enzymes such that "c" refers to the cysteine protease mechanisms and "aspase" refers to their specific ability to cleave after aspartic acid [6]. The initial evidence implicating the role of caspases in cell death came from studies that demonstrated significant sequence homology of these proteases with the cell death gene, ced3, encoding a programmed cell death protein in *C. elegans* [7]. Several members of Ced3/ICE-related family of cysteine proteases have been characterized and shown to play a critical role in apoptotic pathways in mammalian cell lines [1–5]. When transfected to recipient cells, these proteases result in characteristic features of apoptotic cell death including cell shrinkage, chromatin condensation and internucleosomal DNA fragmentation.

Although internucleosomal DNA fragmentation resulting from endonuclease activation is generally considered to be a feature of apoptosis, recent studies have demonstrated that this biochemical feature occurs in cell death traditionally considered a necrotic form of cell death. Thus, in several *in vivo* studies of renal ischemia/reperfusion injury, DNA fragmentation in the kidney cortex [8–10] and other tissues [11–14] has been demonstrated. In addition to these *in vivo* studies, we have demonstrated that freshly isolated tubules subjected to hypoxia/reoxygenation results in DNA strand breaks and nuclear DNA fragmentation (both by an *in situ* technique and by agarose gel electrophoresis), which precedes cell death. Endonuclease inhibitors provided complete protection against DNA damage induced by hypoxia/reoxygenation and partial protection against cell death [15].

Ischemia involves cessation of both exogenous substrate delivery and lack of oxygen, however, its most defining feature is lack of oxygen for ATP synthesis. Thus, metabolic inhibitors of mitochondrial respiratory chain that induce severe ATP depletion characteristic of ischemia have been extensively utilized to study hypoxic injury [16–18], and results in a necrotic form of cell death [16]. In our previous study we demonstrated that this model of chemical hypoxia in LLC-PK_1 cells resulted in multiples of low molecular weight DNA fragments [18] characteristic of endonuclease activation and similar to the findings described above. Similarly, Iwata et al [9] have shown that in hypoxic injury, DNA fragmentation was associated with morphological features of necrosis.

There is no information on the role of caspase proteases in hypoxia-induced DNA damage in any tissue and only a single recent report on their role in hypoxia-induced cell death in neuronal cells [19]. In the present study we first analyzed the caspase activity in antimycin A-induced injury to LLC-PK_1 cells, and then evaluated DNA strand breaks and DNA fragmentation

Key words: DNA damage, caspases, proteases, antimycin A and injury, cell death, enzymes and cell death, apoptosis.

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in the presence and absence of inhibitors of caspases. Further, we determined the effect of these inhibitors on antimycin A-induced cell death.

METHODS

Cell culture

LLC-PK_1 cells obtained from American Type Culture Collection were cultured as in our previous studies [20]. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 20 mM 4-(2-hydroxy ethyl)-1-piperazine ethanesulfonic acid (HEPES) and 2 mM nonessential amino acids. Cultures were maintained in a humidified incubator gassed with 5% CO_2–95% air at 37°C and fed with fresh medium at intervals of 48 to 72 hours. Experiments were performed with cells grown to one to two days postconfluence.

Induction of chemical hypoxia

Chemical hypoxia was induced using a combination of glucose deprivation and mitochondrial electron transport inhibition as previously described [16, 18]. After confluency, cells were washed and incubated with DMEM without serum, glutamine, pyruvate, nonessential amino acids or glucose, containing 3.7 g/liter $NaHCO_3$ and 20 mM HEPES at pH 7.4, either with or without antimycin A for the period of time indicated. In initial studies we determined the optimum exposure time and the suitable concentration of antimycin A. To determine a role of caspases in DNA damage and cell death induced by antimycin A, cells were preincubated with caspase inhibitor I (Ac-Tyr-Val-Ala-Asp-aldehyde, YVAD-CHO) and inhibitor II (Ac-Asp-Glu-Val-Asp-aldehyde, DEVD-CHO) (Bachem Inc., CA, USA, for inhibitors I and II) for 60 minutes, washed and exposed to antimycin A (10 μM). These tetrapeptide inhibitors based on the cleavage sequence irreversibly inhibit select members of the caspase family [21, 22].

Determination of caspase activity

Cells were harvested by centrifugation and the pellets were homogenized with 20 mM HEPES, pH 7.5, containing 10% sucrose, 0.1% CHAPS, 2 mM DTT, 0.1% NP40, 1 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin A. The supernatants obtained after centrifugation were used to determine the enzyme activity. The caspase activity was determined by fluorometric assay using the substrate, Ac-Tyr-Val-Ala-Asp-AMC, which is specifically cleaved by the enzyme to release the fluorescent leaving group, amino-4-methyl coumarin (AMC) [23]. The appropriate amounts of enzyme extracts were incubated with 100 mM HEPES, pH 7.5, containing 10% sucrose, 0.1% CHAPS, 10 mM DTT and 14 μM substrate in a total reaction volume of 0.5 ml. The reaction mixture was incubated for 30 minutes at 25°C. At the end of incubation, the liberated fluorescent group was monitored continuously using a spectrofluorometer (Perkin Elmer) with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. AMC was used as a standard. One unit of enzyme activity is defined as the amount of enzyme required to liberate 2 nM of AMC in 30 minutes. The data for caspase activity are expressed as U/mg protein.

Determination of DNA damage

The residual double strand DNA was measured by the alkaline unwinding assay and determination of ethidium bromide fluorescence according to the method of Birnboim and Jevcak [24], as utilized in our previous studies [15, 18, 20]. Ethidium bromide fluorescence was measured at 520 nm excitation and 590 nm emission using a fluorescence spectrophotometer. Under the conditions employed, ethidium bromide binds preferentially to double strand DNA. Percent double strand DNA (D) was determined by the equation: $\%D = 100 \times [F(P) - F(B)]/[F(T) - F(B)]$, where F(P) is the sample fluorescence, F(T) is the total fluorescence prior to alkaline treatment, and F(B) is the background fluorescence that was obtained from sonicated, alkaline-treated DNA, a condition under which the DNA is completely unwound and represents the fluorescence due to all components other than double stranded DNA.

Detection of DNA fragmentation by agarose gel electrophoresis

Fragmented DNA was isolated from LLC-PK_1 cells as previously described [15, 18, 20, 25]. Cells were collected by centrifugation at 200 g for 10 minutes and the pellets were lysed with 1.0 ml of lysis buffer containing 0.5% Triton X-100, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA for one to two hours at 4°C. The lysates were centrifuged at 16,000 g for 20 minutes to separate the fragmented DNA (supernatant) from the intact DNA (pellet). The resulting supernatant containing fragmented DNA was incubated with proteinase K (200 μg/ml) at 50°C overnight. The DNA was extracted with phenol-chloroform-isoamyl alcohol (50:49:1), followed by precipitation with 0.1 vol of 3 M sodium acetate and 2.5 vol of 100% ethanol. The concentration of nucleic acid in each sample was determined by UV absorbance at 260 nm. The same amount of nucleic acid from each sample was treated with RNAse (20 μg/ml) at 37°C for three hours and subjected to electrophoresis on 1% agarose gel. After electrophoresis the DNA was visualized by UV fluorescence after staining with ethidium bromide.

In situ detection of DNA fragmentation

The terminal deoxynucleotidyl transferase (TdT) nick end labeling (TUNEL) technique was used to detect *in situ* DNA strand breaks as described [26]. Cells were grown to confluency on two-chamber tissue culture slides. Cells were exposed to antimycin A in the absence or presence of caspases inhibitors as described. At the end of the experiment, the cells were fixed in 4% paraformaldehyde and then treated with 1% H_2O_2 in PBS for 10 minutes to remove endogenous peroxidase activity. Following labeling with digoxigenin-labeled nucleotides the cells were treated with peroxidase conjugated anti-digoxigenin antibody and stained with peroxide substrate using the *in situ* ApopTag Plus labeling method (Oncor).

Determination of cell injury and cellular ATP

Cell viability was determined using trypan blue exclusion [27]. Cellular ATP was measured by luciferin/luciferase assay as previously described [16, 17].

Statistics

Results are mean ± SE. Statistical significance was determined by the Student's *t*-test. A P value less than 0.05 was considered to be significant.

RESULTS

We first examined the presence of caspase activity (ICE/Ced3 proteases) in LLC-PK_1 cells. The caspase activity was determined using fluorometric peptide substrate, Ac-Tyr-Val-Ala-Asp-AMC, containing the specific aspartic acid cleavage site for caspase proteases. LLC-PK_1 cells contain caspase activity that is directly proportional to both protein concentration and time of incubation (Fig. 1). This activity is not inhibited by inhibitors of serine proteases (PMSF, aprotinin, SBTI), aspartic proteases (pepstatin A) and metalloproteinases (EDTA, 1,10-phenanthroline) (Table 1). Although ICE and ICE-like proteases are classified under cysteine proteases because of the presence of cysteine in the catalytic site, cysteine protease inhibitors including leupeptin, E-64, E64d and specific calpain peptide inhibitor did not inhibit caspase activity (Table 1). In contrast, specific and competitive tetrapeptide inhibitors of caspase, Ac-Tyr-Val-Ala-Asp-aldehyde (inhibitor I, YVAD-CHO) and Ac-Asp-Glu-Val-Asp-aldehyde (inhibitor II, DEVD-CHO), which are based on the cleavage site aspartic acid and other amino acids required to the left of the cleavage site, markedly inhibited caspase activity (Fig. 2). The use of the specific substrate, lack of inhibition of the activity by other protease inhibitors, and marked inhibition by the caspase inhibitors confirmed the presence of caspase activity in LLC-PK_1 cells and validated the assay utilized for the measurement of the activity.

Caspase activity was increased within five minutes exposure of antimycin A to cells and returned to baseline at longer periods (Fig. 3). We have previously shown that DNA damage in LLC-PK_1 cells occurs in 30 minutes whereas cell death occurs in two hours after antimycin A-induced injury [18]. Taken together, these data indicate that chemical hypoxia-induced increase in caspase activity preceded any evidence of DNA damage and cell death.

We next examined the role of caspase in antimycin A-induced DNA damage utilizing caspase inhibitor I and inhibitor II. LLC-PK_1 cells exposed to 10 μM antimycin A concentration caused marked DNA strand breaks (measured by the alkaline unwinding assay) as shown in Figure 4 and as reported in our previous studies [18]. The caspase inhibitors prevented antimycin A-induced DNA strand breaks in a dose-dependent manner (Fig. 4A). At 50 μM concentration, these inhibitors provided significant protection against antimycin A-induced DNA strand breaks (residual double stranded DNA in control, 94 ± 2%; antimycin A alone, 48 ± 3%; antimycin A + inhibitor I, 93 ± 2%; antimycin A + inhibitor II, 89 ± 5%; $N = 4$, $P < 0.001$, Fig. 4B). Exposure of cells to antimycin A resulted in multiples of low molecular weight DNA fragments as determined by agarose gel electrophoresis (Fig. 5), and as shown in our previous studies [18]. The inhibition of caspase activity provided marked protection against antimycin A-induced DNA fragmentation (Fig. 5). We also examined the effect of caspase inhibitors I and II on antimycin A-induced DNA damage by *in situ* in cell nuclei utilizing the TUNEL technique. As shown in Figure 6, a significant number of nuclei of antimycin A treated cells were positively stained for DNA strand breaks

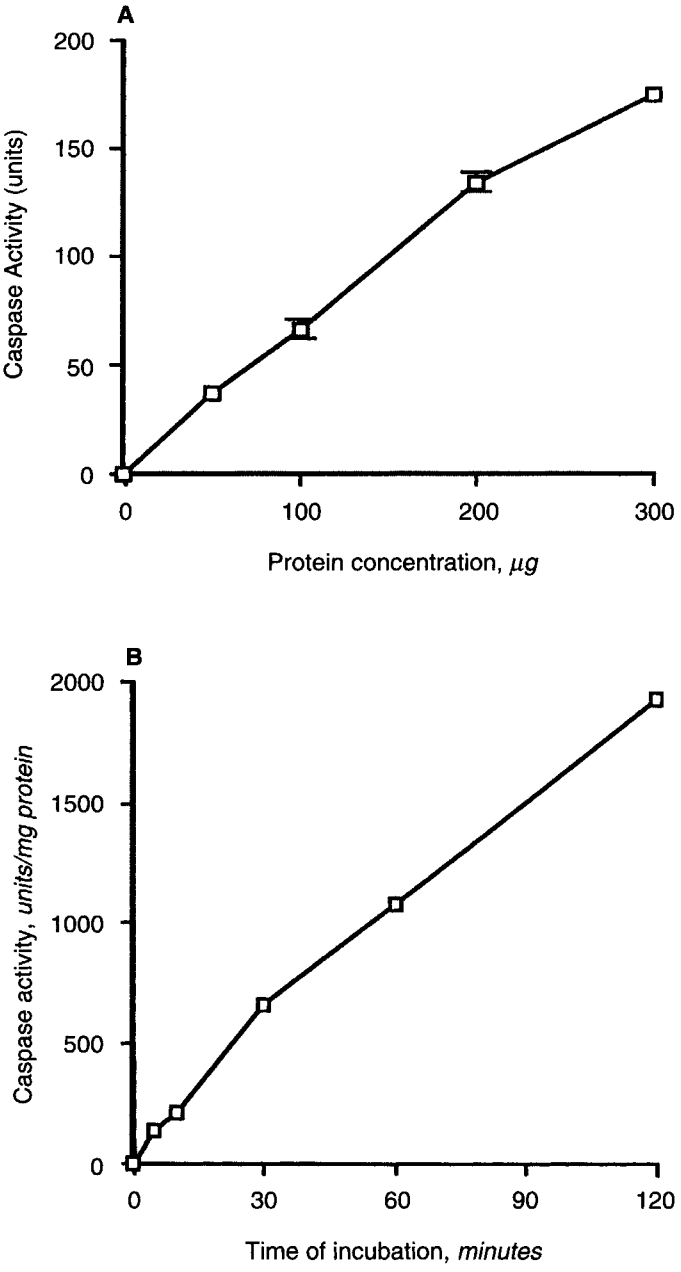

Fig. 1. Protein dependence and time course of caspase activity. (*A*) Various amounts of cell extracts were incubated in an assay mixture containing fluorometric substrate, Ac-Tyr-Val-Ala-Asp-AMC (14 μM), 20 mM HEPES, pH 7.5, containing 10% sucrose, 0.1% CHAPS, 2 mM DTT, 0.1% NP40, 1 mM EDTA, 1 mM PMSF, 1 μg/ml leupeptin and 1 μg/ml pepstatin A in a total volume of 0.5 ml for 30 minutes at 25°C. At the end of incubation, the liberated AMC was determined using a spectrofluorometer with an excitation wavelength of 380 nm and an emission wavelength of 460 nm. Results are mean ± SEM, $N = 4$. (*B*) Cell extract (300 μg) was added to assay mixture and incubated for various times shown. The values are an average of two determinations.

compared to the corresponding controls that showed no detectable *in situ* DNA nick-end labeling. In cells exposed to antimycin A, the morphology of the cell nuclei showing positive staining (brown) for DNA strand breaks was relatively preserved (Fig. 6). The cells treated with the caspase inhibitors did not show distinct

Table 1. Effect of proteinase inhibitors on caspase activity

Inhibitor	Concentration	Enzyme activity *% of control*
PMSF	0.5 mM	100 ± 2
Aprotinin	10 μg/ml	101 ± 2
SBTI	0.5 mM	88 ± 1
Leupeptin	10 mg/ml	101 ± 1
Pepstatin A	10 μg/ml	97 ± 2
EDTA	0.5 mM	85 ± 1
1,10-Phenanthroline	1 mM	74 ± 1
E-64	50 μM	96 ± 1
E-64d	20 μM	87 ± 1
Calpain peptide inhibitor	20 μM	76 ± 1

The enzyme extract (300 μg) was preincubated for 10 minutes with various amounts of inhibitors before the addition of the substrate. The incubations were continued for 30 minutes and the liberated AMC product was determined as described in the **Methods** section. The activity in control was considered 100%. Values are means ± SE, $N = 4$.

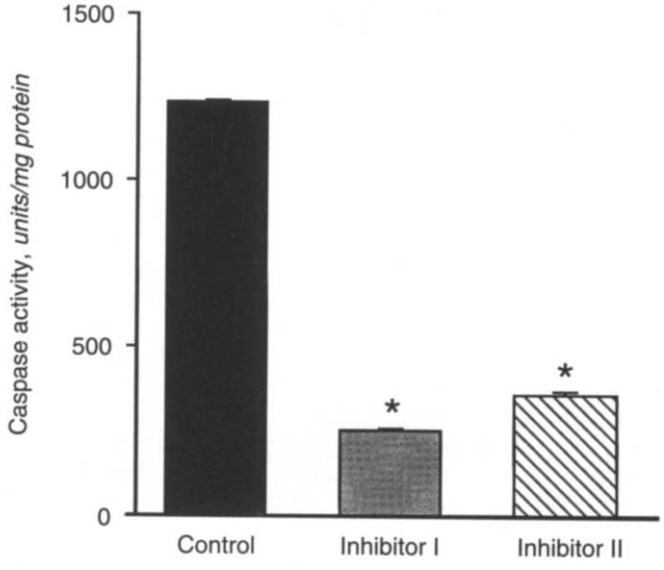

Fig. 2. Effect of specific caspase inhibitors on the caspase activity in LLC-PK$_1$ cells. The cell extracts (200 μg) were preincubated with 50 μM each of caspase inhibitors I (YVAD-CHO) and II (DEVD-CHO) before the addition of the caspase substrate, Ac-Tyr-Val-Ala-Asp-AMC. The reaction mixture was prepared, incubated for 60 minutes and assayed as described in the **Methods** section. Results are mean ± SE, $N = 3$. *$P <$ 0.001 compared to control.

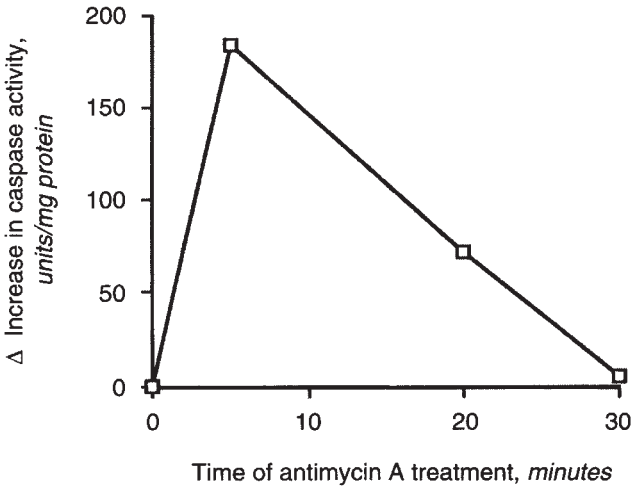

Fig. 3. Time course effect of antimycin A-induced injury on caspase activity in LLC-PK$_1$ cells. The caspase activity was determined at various times of antimycin A exposure as described in the **Methods** section. The net increase in caspase activity was obtained by subtracting the values of control cells from the corresponding values of antimycin A treated cells. Results are mean ± SE, $N = 3$. $P < 0.001$ compared with control cells.

pattern of staining for DNA strand breaks, indicating that the inhibitors afford strong protection against antimycin A-induced DNA damage. We considered the possibility that the caspase inhibitors could block the cellular uptake of antimycin A. If this was the case, then the fall in ATP level induced by antimycin A would be reduced in the presence of caspase inhibitors. There was a similar and dramatic fall in ATP levels in the presence of caspase inhibitors. Cellular ATP declined from 31.0 ± 0.05 to 1.5 ± 0.05 nmol/mg protein in cells treated with antimycin A alone ($N = 2$). There was no difference in cellular ATP level between cells exposed to antimycin A alone and those pretreated with the caspase inhibitor I (1.5 ± 0.05 nmol/mg protein), or inhibitor II (1.4 ± 0.05 nmol/mg protein), suggesting that the protective effect of caspase inhibitors was not due to the effect on antimycin A uptake.

Since antimycin A-induced injury to LLC-PK$_1$ cells results in significant cell death [18], we determined whether caspase inhibitors provide protection against antimycin A-induced cell death. Treatment of cells with the tetrapeptide caspase inhibitors I and II prevented hypoxia-induced cell death as measured by trypan blue exclusion in a dose-dependent manner (Fig. 7A). At 50 μM concentration, these inhibitors significantly prevented antimycin A-induced cell death (control, 6 ± 1%; antimycin A alone, 40 ± 1%; antimycin A + inhibitor I, 16 ± 1%; antimycin A + inhibitor II, 16 ± 1%; $N = 4$ to 7, $P < 0.001$, Fig. 7B). We confirmed that the caspase inhibitors did not prevent the cellular uptake of the trypan blue dye when added along with the dye (antimycin A alone, 42 ± 1%; antimycin A + inhibitor I at 50 μM, 41 ± 2%; antimycin A + inhibitor II at 50 μM, 42 ± 1%; $N = 3$).

DISCUSSION

In the present study we have demonstrated that LLC-PK$_1$ cells contain caspase activity that is not inhibited by inhibitors of serine proteases (PMSF, aprotinin SBTI), aspartic proteases (pepstatin A), and metalloproteinases (EDTA, 1,10-phenanthroline). Caspase activity is also not inhibited by other cysteine protease inhibitors including leupeptin, E-64, E64d and a specific calpain peptide inhibitor. However, this activity is markedly inhibited by peptide inhibitors of caspase proteases, YVAD-CHO and DEVD-CHO, indicating the presence of caspases in LLC-PK$_1$ cells. We have shown that antimycin A treatment results in rapid increase in caspase activity prior to any evidence of DNA damage and cell death.

Although most of the recent studies related to hypoxia/reoxygenation *in vitro* and ischemia/reperfusion *in vivo* injury provide evidence for internucleosomal DNA fragmentation and endonuclease activation, there is no information on the role of caspase proteases in antimycin A-induced DNA damage in any tissue. In the present study we have utilized some of the most potent and

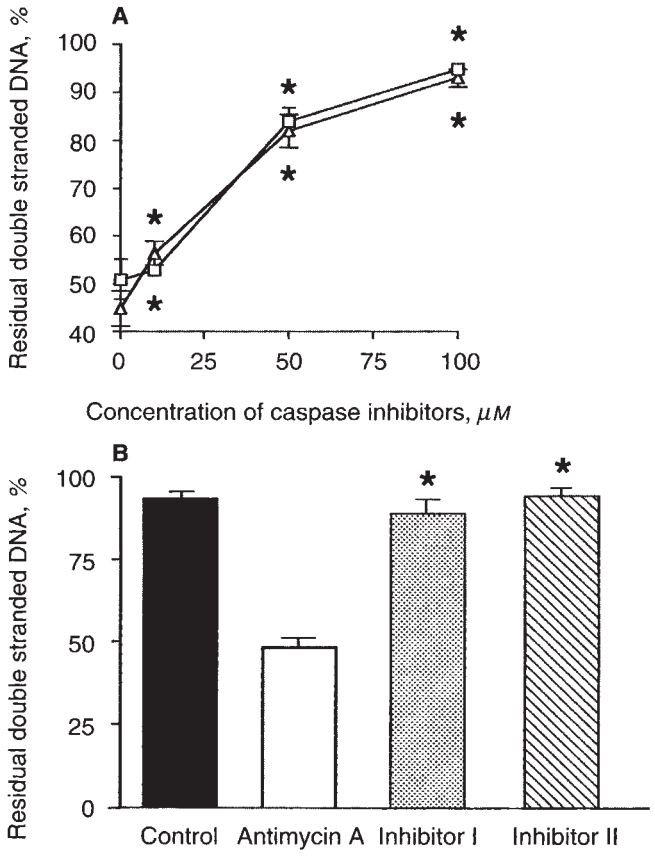

Fig. 4. (*A*) Effect of increasing concentration of caspase inhibitors on antimycin A-induced DNA strand breaks in LLC-PK$_1$ cells. Cells were preincubated for 60 minutes with increasing concentrations of inhibitor I or II prior to induction of chemical hypoxia with antimycin A (10 μM) for an additional 60 minutes. Residual double stranded DNA was determined by alkaline unwinding assay as described in the **Methods** section. Symbols are: (□) inhibitor I (YVAD-CHO); (△) inhibitor II (DEVD-CHO). Results are mean ± SE, $N = 4$. $^*P < 0.001$ compared to cells exposed to antimycin A alone. **(*B*) Effect of caspase inhibitors on antimycin A-induced DNA strand breaks in LLC-PK$_1$ cells.** Cells were preincubated with 50 μM concentration of caspase inhibitor I or inhibitor II for 60 minutes prior to induction of chemical hypoxia with antimycin A (10 μM) for an additional 60 minutes. Results are mean ± SE, $N = 4$. $^*P < 0.001$ compared to cells exposed to antimycin A alone. Symbols are: (■) control; (□) antimycin A alone; (▨) antimycin A + inhibitor I; (▧) antimycin A + inhibitor II.

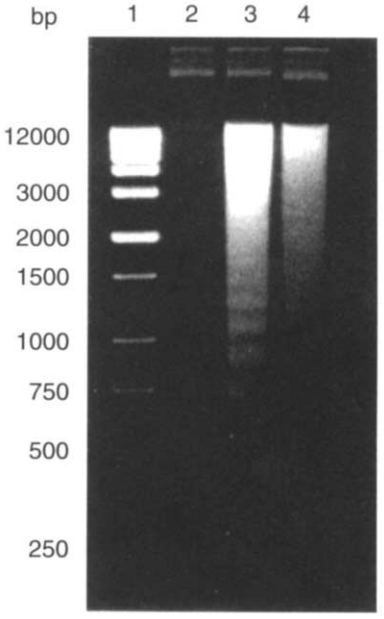

Fig. 5. Effect of caspase inhibition on antimycin A-induced DNA fragmentation. Cells were exposed to antimycin A (10 μM) for 120 minutes in the absence and presence of 50 μM caspase inhibitor I (YVAD-CHO). The fragmented DNA was isolated from each sample and subjected to 1% agarose gel electrophoresis. Lanes are: (1) molecular size marker, (2) control, (3) antimycin A alone, (4) antimycin A + caspase inhibitor I. The data depicted are representative of four experiments.

reversible competitive inhibitors of caspases, YVAD-CHO and DEVD-CHO, to investigate the role of caspases in antimycin A-induced injury. These peptide inhibitors are based on the cleavage site of aspartic acid and other amino acids required to the left of the cleavage site [21, 22, 28] for caspases. We have shown that these cleavage-site directed peptide inhibitors prevented DNA strand breaks as revealed by an alkaline unwinding assay, and internucleosomal DNA fragmentation revealed by agarose gel electrophoresis in antimycin A-induced hypoxic injury to LLC-PK$_1$ cells. In addition, *in situ* studies based on specific labeling of DNA breaks in nuclei with TdT and subsequent immunohistochemical staining of fragmented DNA in cells further demonstrate that the peptide inhibitors provide protection against DNA fragmentation in antimycin A-induced injury. Thus, the protective effects of the peptide inhibitors strongly suggest the role of caspases in mediating DNA damage in antimycin A-induced injury. To our knowledge, this is the first report on the protective effect of caspase protease inhibitors on DNA damage in chemical hypoxic injury to cells.

The internucleosomal DNA fragmentation observed in antimycin A-induced injury can result from the cleavage of chromatin proteins (histones and nuclear matrix proteins) followed by cleavage of exposed DNA by endonucleases. At present it is not known which nuclear matrix proteins or other protein substrates are involved in hypoxic/ischemic injury. However, recent studies have demonstrated that DNA fragmentation observed in apoptotic cell death in several mammalian cell lines is accompanied by the cleavage of several potential target substrates. A number of target proteins for caspases have been identified including nuclear lamin subunits [29–31], the nuclear enzyme Poly (ADP-ribose) polymerase [21, 32, 33] and DNA-dependent protein kinase [34], both involved in aspects of DNA damage sensing and repair [35, 36], U1 small nuclear ribonucleoprotein [37], and actin [38]. In ischemic or hypoxic injury, caspase proteases may play a fundamental role in DNA damage and subsequent cell death presumably through their proteolytic action on proteins essential for cellular repair and other target proteins, including the proteins required for the stability of DNA itself.

In parallel studies we also demonstrated that in addition to prevention of internucleosomal DNA cleavage, caspase inhibitors protected against cell death in antimycin-induced hypoxic injury to LLC-PK$_1$ cells in a dose dependent manner. While this work was in progress, a pertinent study consistent to our results by Shimuzu et al [19] demonstrated that caspase inhibitors prevented cell death in rat pheochromocytoma cells (PC12 cells) exposed to cyanide, rotenone or antimycin A. Thus, it is apparent from these

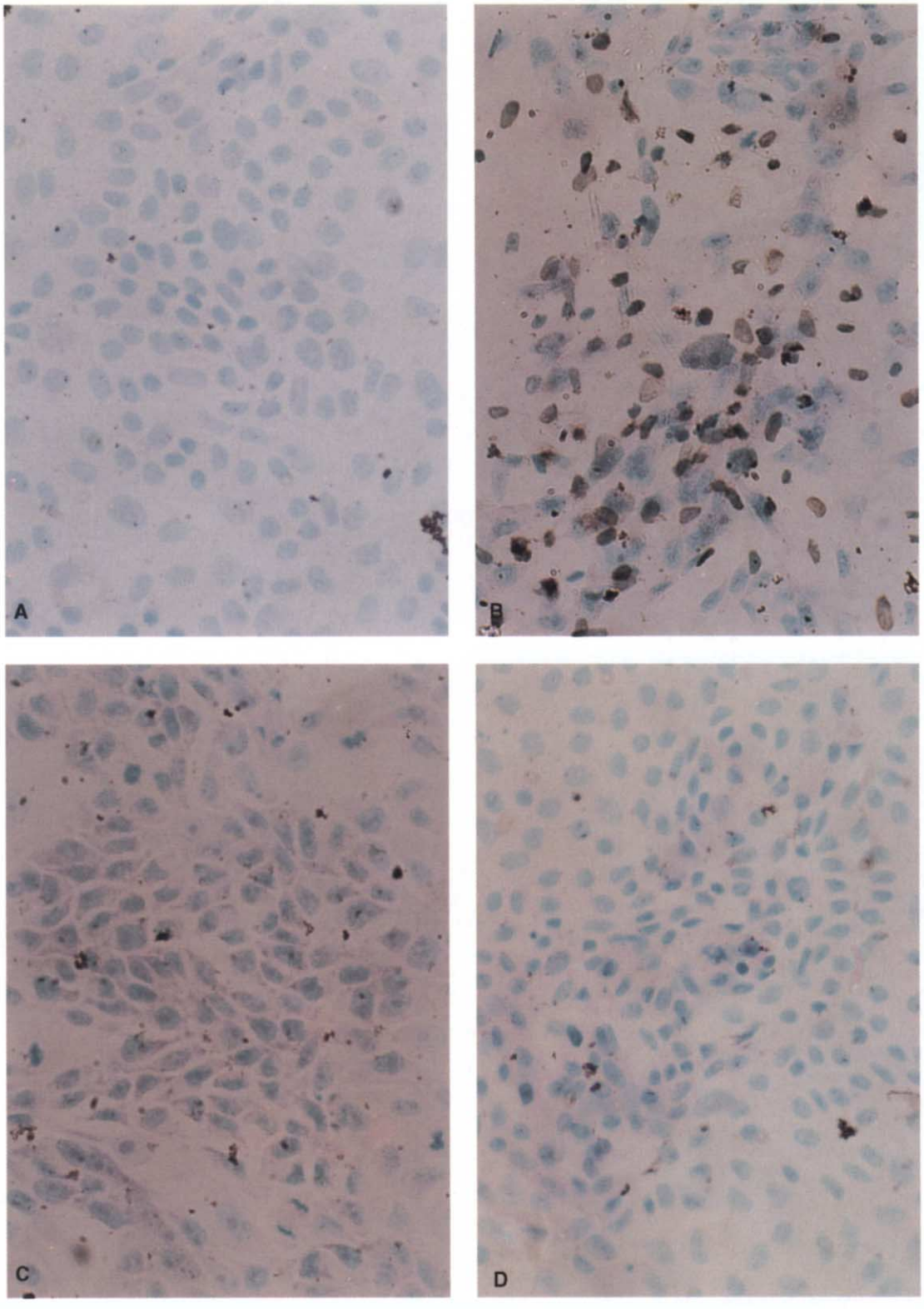

Fig. 6. Effect of caspase inhibitors on *in situ* chemical hypoxia-induced DNA strand breaks in LLC-PK$_1$ cells. Cells were grown to confluence on two-chamber tissue culture slides. Cells were exposed to antimycin A for 120 minutes in the absence or presence of 50 μM caspase inhibitor I or inhibitor II. The cells were analyzed for *in situ* DNA strand breaks by terminal deoxynucleotidyl transferase (TdT)/digoxigenin dUTP nick end labeling (TUNEL) technique as described in the **Methods** section. (*A*) Control. (*B*) Cells treated with antimycin A alone. (*C*) Cells exposed to antimycin A plus caspase inhibitor I. (*D*) Cells exposed to antimycin A plus caspase inhibitor II.

studies that cell injury induced by inhibition of mitochondrial respiratory chain involves the participation of caspases. In addition to caspases, other proteases may also be involved during hypoxic injury. A recent study has shown that calpain activity is increased by 7.5 minutes of hypoxia to proximal tubules and the cysteine protease inhibitor, N-benzyloxycarbonyl-Val-Phe-methyl

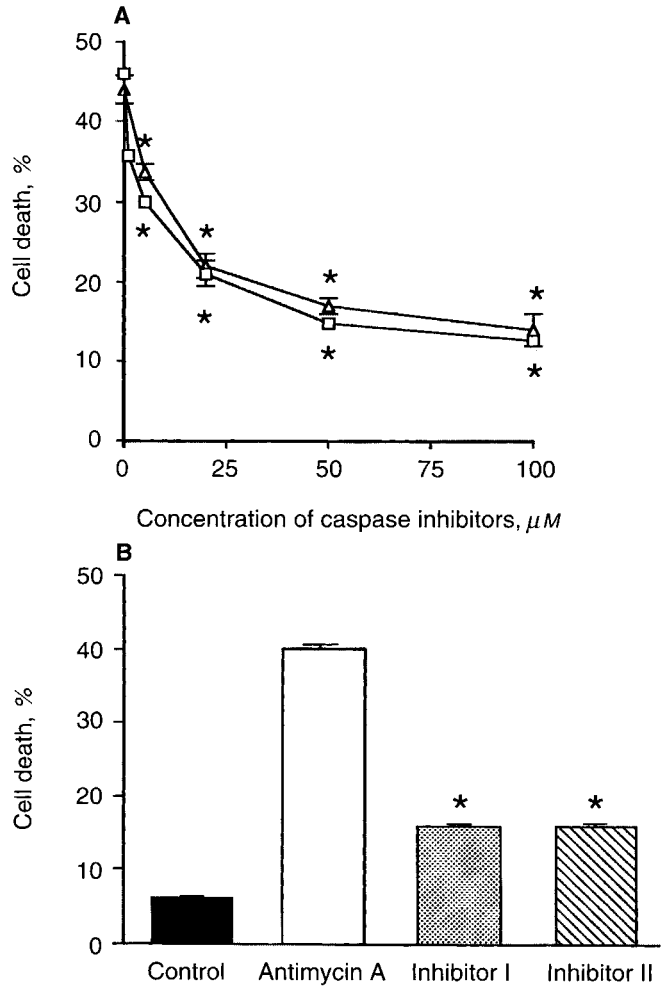

Fig. 7. (*A*) Dose-dependent effect of caspase inhibitors on antimycin A-induced cell death in LLC-PK$_1$ cells. Cells were preincubated for 60 minutes with increasing concentrations of inhibitor I or II prior to induction of chemical hypoxia with antimycin A (10 μM) for an additional 60 minutes. Cell death was measured by trypan blue exclusion. Symbols are: (□) inhibitor I; (△) inhibitor II. Results are mean ± SE, $N = 4$, $^*P < 0.001$, compared to cells exposed to antimycin A alone. **(*B*) Effect of caspase inhibitors on antimycin A-induced cell death in LLC-PK$_1$ cells.** Cells were preincubated with 50 μM concentration of caspase inhibitor I or inhibitor II for 60 minutes prior to induction of chemical hypoxia with antimycin A (10 μM) for an additional 60 minutes. Results are mean ± SE, $N = 4$ to 7; $^*P < 0.001$, compared to cells exposed to antimycin A alone. Symbols are: (■) control; (□) antimycin A alone; (▨) antimycin A + inhibitor I; (▧) antimycin A + inhibitor II.

ester, markedly decreases cell death as determined by LDH release [39].

The ability of the caspase protease inhibitors to prevent DNA damage and cell death raises the question of which caspase proteases are involved in antimycin A-induced hypoxic injury. At present, several members of the caspase related family of cysteine proteases have been characterized including caspase-2 (Nedd2/ICH1) [40, 41], caspase-3 (Yama/CPP32/Apopain) [21, 32, 42], caspase-4 (Tx/ICH2/ICE rel-II) [43–45], caspase-5 (ICE rel-III) [43], caspase-6 (Mch2) [46], caspase-7 (Mch3/ICE-LAP3) [47], caspase-8, (ICE-LAP6/Mch6) [48], caspase-9 (FLICE/Mach/Mch5) [49], caspase-10 (Mch4) [50], and caspase-11 (ICH-3) [51].

In future studies the mechanism(s) of activation of the caspase(s), the potential targets as well the particular members of the caspase family involved in these processes will need to be identified.

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REFERENCES

1. Martin SJ, Green DR: Protease activation during apoptosis: Death by a thousand cuts? *Cell* 82:349–352, 1995
2. Fraser A, Evan G: A license to kill. *Cell* 85:781–786, 1996
3. Thornberry NA, Molineaux SM: Interleukin-1β converting enzyme: A novel cysteine protease required for IL-1β production and implicated in programmed cell death. *Protein Sci* 4:3–12, 1995
4. Vaux DL, Strasser A: The molecular biology of apoptosis. *Proc Natl Acad Sci USA* 93:2239–2244, 1996
5. Kumar S: ICE-like proteases in apoptosis. *TIBS* 20:198–202, 1995
6. Alnemri ES, Livingston DJ, Nicholson DW, Salvesen G, Thornberry NA, Wong WW, Yuan J: Human ICE/CED-3 protease nomenclature. (abstract) *Cell* 87:171, 1996
7. Yuan J, Shaham S, Ledoux S, Ellis HM, Horvitz HR: The *C. elegans* cell death gene ced-3 encodes a protein similar to mammalian interleukin-1β-converting enzyme. *Cell* 75:641–652, 1993
8. Beeri R, Symon Z, Brezis M, Ben-Sasson SA, Baehr PH, Rosen S, Zager RA: Rapid DNA fragmentation from hypoxia along the thick ascending limb of rat kidneys. *Kidney Int* 47:1806–1810, 1995
9. Iwata M, Myerson D, Torok-Storb B, Zager RA: An evaluation of renal tubular DNA laddering in response to oxygen deprivation and oxidant injury. *J Am Soc Nephrol* 5:1307–1313, 1994
10. Schumer M, Colombel MC, Sawczuk IS, Gobe G, Connor J, O'Toole KM, Olsson CA, Wise GJ, Buttyan R: Morphologic, biochemical, and molecular evidence of apoptosis during the reperfusion phase after brief periods of renal ischemia. *Am J Pathol* 140:831–838, 1992
11. Gottlieb RA, Burleson KO, Kloner RA, Babior BM, Engler RL: Reperfusion injury induces apoptosis in rabbit cardiomyocytes. *J Clin Invest* 94:1621–1628, 1994
12. Didenko VV, Wang X, Yang L, Hornsby PJ: Expression of p21WAF1/CIP1/SKI1 and p53 in apoptotic cells in the adrenal cortex and induction by ischemia/reperfusion injury. *J Clin Invest* 97:1723–1731, 1996
13. Macmanus JP, Hill IE, Huang ZG, Rasquinha I, Xue D, Buchan AM: DNA damage consistent with apoptosis in transient focal ischaemic neocortex. *Neuroreport* 5:493–496, 1994
14. Tanaka M, Ito H, Adachi S, Akimoto H, Nishikawa T, Kasjima T, Marumo F, Hiroe M: Hypoxia induces apoptosis with enhanced expression of Fas antigen messenger RNA in cultured neonatal rat cardiomyocytes. *Circ Res* 75:426–433, 1994
15. Ueda N, Walker PD, Hsu S-M, Shah SV: Activation of a 15-kDa endonuclease in hypoxia/reoxygenation injury without morphologic features of apoptosis. *Proc Natl Acad Sci USA* 92:7202–7206, 1995
16. Venkatachalam MA, Patel YJ, Kreisberg JI, Weinberg JM: Energy thresholds that determine membrane integrity and injury in a renal epithelial cell line (LLC-PK$_1$). Relationships to phospholipid degradation and unesterifed fatty acid accumulation. *J Clin Invest* 81:745–758, 1988
17. Molitoris BA, Geerdes A, McIntosh JR: Dissociation and redistribution of Na^+,K^+-ATPase from its surface membrane actin cytoskeletal complex during cellular ATP depletion. *J Clin Invest* 88:462–469, 1991
18. Hagar H, Ueda N, Shah SV: Endonuclease induced DNA damage and cell death in chemical hypoxic injury to LLC-PK$_1$ cells. *Kidney Int* 49:355–361, 1996

19. Shimizu S, Eguchi Y, Kamiike W, Waguri S, Uchiyama Y, Matsuda H, Tsujimoto Y: Retardation of chemical hypoxia-induced necrotic cell death by Bcl-2 and ICE inhibitors: Possible involvement of common mediators in apoptotic and necrotic signal transductions. *Oncogene* 12:2045–2050, 1996
20. Ueda N, Shah SV: Endonuclease-induced DNA damage and cell death in oxidant injury to renal tubular epithelial cells. *J Clin Invest* 90:2593–2597, 1992
21. Nicholson DW, Ali A, Thornberry NA, Vaillancourt JP, Ding CK, Gallant M, Gareau Y, Griffin PR, Labelle M, Lazebnik YA, Munday NA, Raju SM, Smulson ME, Yamin TT, Yu VL, Miller DK: Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* 376:37–43, 1995
22. Thornberry NA, Bull HG, Calaycay JR, Chapman KT, Howard AD, Kostura MJ, Miller DK, Molineaux SM, Weidner JR, Aunins J, Elliston KO, Ayala JM, Casano FJ, Chin, J, Ding GJF, Egger LA, Gaffney EP, Limjuco G, Palyha OC, Raju SM, Rolando AM, Salley JP, Yamin TT, Lee TD, Shivel JE, MacCross M, Mumford RA, Schmidt JA, Tocci MJ: A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. *Nature* 356:768–774, 1992
23. Thornberry NA: Interleukin-1 beta converting enzyme. *Meth Enzymol* 244:615–631, 1994
24. Birnboim HC, Jevcak JJ: Fluorometric method for rapid detection of DNA strand breaks in human white blood cells produced by low doses of radiation. *Cancer Res* 41:1889–1892, 1981
25. Hockenbery D, Nunez G, Milliman C, Schreiber RD, Korsmeyer SJ: Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 348:334–336, 1990
26. Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol* 119:493–501, 1992
27. Walker PD, Shah SV: Hydrogen peroxide cytotoxicity in LLC-PK_1 cells: A role for iron. *Kidney Int* 40:891–898, 1991
28. Dolle RE, Hoyer D, Prasad CV, Schmidt SJ, Helaszek CT, Miller RE, Ator MA: P1 aspartate-based peptide alpha-((2,6-dichlorobenzoyl)oxy)methyl ketones as potent time-dependent inhibitors of interleukin-1 beta-converting enzyme. *J Med Chem* 37:563–564, 1994
29. Orth K, Chinnaiyan AM, Garg M, Froelich CJ, Dixit VM: The CED-3/ICE-like portease Mch2 is activated during apoptosis and cleaves the death substrate lamin A. *J Biol Chem* 271:16443–16446, 1996
30. Oberhammer FA, Hochegger K, Froschl G, Tiefenbacher R, Pavelka M: Chromatin condensation during apoptosis is accompanied by degradation of lamin A+B without enhanced activation of cdc2 kinase. *J Cell Biol* 126:827–837, 1994
31. Neamati N, Fernandez A, Wright S, Kiefer J, McConkey DJ: Degradation of lamin B1 precedes oligonucleosomal DNA fragmentation in apoptotic thymocytes and isolated thymocyte nuclei. *J Immunol* 154:3788–3795, 1995
32. Tewari M, Quan LT, O'Rourke K, Desnoyers S, Zeng Z, Beidler DR, Poirier GG, Salvesen GS, Dixit VM: Yama/CPP32b, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. *Cell* 81: 801–809, 1995
33. Lazebnik YA, Kaufmann SH, Desnoyers S, Poirier GG, Earnshaw WC: Cleavage of poly(ADP-ribose) polymerase by a proteinase with properties like ICE. *Nature* 371:346–347, 1994
34. Casciola-Rosen LA, Anhalt GJ, Rosen A: DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. *J Exp Med* 182:1625–1634, 1995
35. Satoh MS, Lindahl T: Role of poly(ADP-ribose) formation in DNA repair. *Nature* 356:358–358, 1992
36. Morozov VE, Falzon M, Anderson CW, Kuff EL: DNA-dependent protein kinase is activated by nicks and larger single-stranded gaps. *J Biol Chem* 269:16684–16688, 1994
37. Casciola-Rosen LA, Miller DK, Anhalt GJ, Rosen A: Specific cleavage of the 70-kDa protein component of the U1 small nuclear ribonucleoprotein is a characteristic biochemical feature of apoptotic cell death. *J Biol Chem* 269:30757–30760, 1994
38. Kayalar C, Ord T, Testa MP, Zhong LT, Bredesen DE: Cleavage of actin by interleukin 1 beta-converting enzyme to reverse DNase I inhibition. *Proc Natl Acad Sci USA* 93:2234–2238, 1996
39. Edelstein CL, Wieder ED, Yaqoob MM, Gengaro PE, Burke TJ, Nemenoff RA, Schrier RW: The role of cysteine proteases in hypoxia-induced rat renal proximal tubular injury. *Proc Natl Acad Sci USA* 92:7662–7666, 1995
40. Kumar S, Kinoshita M, Noda M, Copeland N, Jenkins N: Induction of apoptosis by the mouse Nedd2 gene, which encodes a protein similar to the product of the Caenorhabditis elegans cell death gene ced-3 and the mammalian IL-1 beta-converting enzyme. *Genes Dev* 8:1613–1626, 1994
41. Wang L, Miura M, Bergeron L, Zhu H, Yuan J: Ich-1, an Ice/ced-3-related gene, encodes both positive and negative regulators of programmed cell death. *Cell* 78:739–750, 1994
42. Fernandes-Alnemri T, Litwack G, Alnemri ES: CPP32, a novel human apoptotic protein with homology to *Caenorhabditis elegans* cell death protein Ced-3 and mammalian interleukin-1 β-converting enzyme. *J Biol Chem* 269:30761–30764, 1994
43. Munday NA, Vaillancourt JP, Ali A, Casano FJ, Miller DK, Molineaux SM, Yamin T-T, Yu VL, Nicholson DW: Molecular cloning and pro-apoptotic activity of $ICE_{rel}II$ and $ICE_{rel}III$, members of the ICE/CED-3family of cysteine proteases. *J Biol Chem* 270: 15870–15876, 1995
44. Faucheu C, Diu A, Chan AWE, Blanchet A-M, Miossec C, Herve F, Collard-Dutilleul V, Gu Y, Aldape RA, Lippke JA, Rocher C, Su MS-S, Livingston DJ, Hercend T, Lalanne J-L: A novel human protease similar to the interleukin-1β converting enzyme induces apoptosis in transfected cells. *EMBO J* 14:1914–1922, 1995
45. Kamens J, Paskind M, Hugunin M, Talanian RV, Allen H, Banach D, Bump N, Hackett M, Johnston CG, Li P, Mankovich JA, Terranova M, Ghayur T: Identification and characterization of ICH-2, a novel member of the interleukin-1β-converting enzyme family of cysteine proteases. *J Biol Chem* 270:15250–15256, 1995
46. Fernandes-Alnemri T, Litwack G, Alnemri ES: *Mch2*, a new member of the apoptotic *Ced-3/Ice* cysteine protease gene family. *Cancer Res* 55:2737–2742, 1995
47. Duan H, Chinnaiyan AM, Hudson PL, Wing JP, He WW, Dixit VM: ICE-LAP3, a novel mammalian homologue of the Caenorhabditis elegans cell death protein Ced-3 is activated during Fas- and tumor necrosis factor-induced apoptosis. *J Biol Chem* 271:1621–1625, 1996
48. Duan H, Orth K, Chinnaiyan AM, Poirier GG, Froelich CJ, He W-W, Dixit VM: ICE-LAP6, a novel member of the ICE/Ced-3gene family, is activated by the cytotoxic T cell protease granzyme B. *J Biol Chem* 271:16720–16724, 1996
49. Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A, Ni J, Scaffidi C, Bretz JD, Zhang M, Gentz R, Mann M, Krammer PH, Peter ME, Dixit VM: FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* 85:817–827, 1996
50. Fernandes-Alnemri T, Armstrong RC, Krebs J, Srinivasula SM, Wang L, Bullrich F, Fritz LC, Trapani JA, Tomaselli KJ, Litwack G, Alnemri ES: In vitro activation of CPP32 and Mch3 by Mch4, a novel human apoptotic cysteine protease containing two FADD-like domains. *Proc Natl Acad Sci USA* 93:7464–7469, 1996
51. Wang S, Miura M, Jung Y-K, Zhu H, Gagliardini V, Shi L, Greenberg AH, Yuan J: Identification and characterization of Ich-3, a member of the interleukin-1β converting enzyme (ICE)/Ced-3 family and an upstream regulator of ICE. *J Biol Chem* 271:20580–20587, 1996